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Original article

Intracerebral vaccination suppresses the spread of rabies virus in the mouse brain

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Abstract

To investigate the efficacy of intracerebral (IC) immunization in preventing viral spread in the brain, we immunized mice with inactivated rabies virus via the subcutaneous (SC) or IC route, followed by administration of a lethal dose of rabies virus (challenge virus standard strain), directly into the brains of immunized mice. Progressive paralytic neurological signs were observed in control and 75% of SC immunized mice, whereas only 20% of IC immunized mice exhibited symptoms. Neutralizing antibody titers in blood plasma were significantly elevated in SC and IC immunized mice, with the highest levels seen in IC immunized mice. Analysis of whole brain lysates revealed a strong induction of immunoglobulin in the brains of IC immunized mice that had virus neutralizing activity. Histopathological examination of brain tissue revealed mild encephalitis and disseminated viral antigen in control and SC immunized mice, but rare in IC immunized mice. These results suggest that IC immunization induces a preventive humoral immune response against intracerebrally inoculated rabies virus. Induction of neutralizing antibody in cerebrospinal fluid represents a putative therapeutic measure for the treatment of rabid animals and humans.

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Keywords: Rabies virus; Intracerebral immunization; Intrathecal immunization; Neutralizing antibody

1. Introduction

Rabies is one of the classical lethal zoonotic diseases in mammals, including humans. Although rabies is a major public health concern in Asia, Africa and Latin America, and is reemerging in North America as bat rabies [1], no effective treatment is currently available in rabid humans or animals showing neurological symptoms. The virus invades the central nervous system (CNS) from peripheral nerves and induces furious or paralytic clinical symptoms in the infected host. In neurons, the virus replicates slowly with or without minimal cytolysis and inflammation [2].

The major source of rabies virus (RV) infection is through a bite by a rabid animal. Proper post-exposure vaccination and injection of rabies immunoglobulin are effective in preventing

rabies [3,4], but is costly and is not commonly available in many countries [5,6]. Furthermore, the protective effects of vaccination administered through ordinary routes (SC or intramuscular) do not prevent transneuronal spread of the pathogen, since antibodies in the blood will neutralize viruses only before they enter neurons or the nervous system [7–9]. Thus, more effective preventive and therapeutic measures against this neurotropic virus will require strategies for blocking transmission and/or transport into and within the nervous systems.

Intrathecal immunization is a method of injecting antigens directly into subarachnoid spaces such as ventricle or brain parenchyma [10–12], and has been shown to induce antigen-specific antibodies not only in the blood serum, but also in the cerebrospinal fluid (CSF). Previously, we demonstrated complete protection against pseudorabies virus and RV challenge in peripheral tissue through IC immunization in mice [13,14]. Significantly higher levels of neutralizing antibody were induced in the blood of IC immunized mice as compared

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to SC immunized mice; however the site of neutralization and clearance of inoculated virus in IC immunized mice was not determined. Here, we inoculated a lethal dose of live RV directly into the brains of IC immunized mice to assess the protective effects of IC immunization against rabies in the CNS.

2. Materials and methods

2.1. Mice and immunization

Sixty-two 6-week-old female ICR mice were prepared from Nihon Clea Inc. (Tokyo, Japan) and divided into 3 groups: negative control (PBS inoculation into brain), SC immunization, and IC immunization. For the SC immunization group, 30 μ l of tissue-cultured inactivated rabies virus (Nisseiken Co., Tokyo, Japan) were inoculated into the dorsal subcutaneous tissue of the mice. For the negative control and IC immunization group, two-step syringe needles (Top Co., Tokyo, Japan) were placed into the right cerebral hemisphere (near caudal 2 mm, lateral 2.5 mm from bregma, and 3 mm in depth). 30 μ l of PBS or inactivated rabies virus were inoculated carefully into the lateral ventricle of the cerebrum under isoflurane inhalation anesthesia. Immunizations were performed twice at 2-week intervals. Seven days after second immunization, four mice of each group were euthanized following blood collection. All mice were performed systemic perfusion from heart with enough volume of sterile saline and the brains were collected.

2.2. Virus challenge and sample collection

One week after the second immunization, all mice of each group were inoculated with 600 fluorescent focus-forming units of live RV, challenging virus standard, into the left lateral ventricle of the brain in the same manner as vaccination. Clinical signs, including neurological symptoms and changes in body weight were recorded for 15 days following virus inoculation. At 3 and 5 through 10 days post virus inoculation (dpi), three to six mice from each group were euthanized following blood collection. Brain, liver, spleen, kidney, heart and lungs were processed for histological examination and pieces of brain tissues were frozen at -80°C for Western blot analysis. All animal experiments were carried out with the approval of the committee of Laboratory Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University and were consistent with the Association for Assessment and Accreditation of Laboratory Animal Care International standards.

2.3. Virus neutralizing antibody titer

RV neutralizing antibody titers in blood plasma were measured using the rapid fluorescent focus inhibition test (RFFIT) [15,16]. Plasma samples were first incubated for 30 min at 56°C to inactivate complement complexes, and then diluted two-fold and mixed with an approximately 50-fold

volume containing a 50% focus-forming dose of live RV. The mixture was used to inoculate mouse neuroblastoma cells (NA) and virus antigen was detected by indirect immunofluorescent staining 72 h after inoculation. The minimum dilution ratio showing immune-positive signals was defined as the neutralizing activity of the sample. The results were transformed to geometric mean titers (GMT). Comparison of the calculated GMTs for the groups was done using the Tukey–Kramer multiple comparison test ($p < 0.05$). Immunoglobulins isolated from each brain were mixed with 30 focus-forming dose of live RV and the mixture was inoculated NA. Virus antigens were detected and counted by indirect immunofluorescent staining 72 h after inoculation. The numbers of immunofluorescence foci were compared to each group of mice.

2.4. Brain lysis and Western blot analysis

Whole brain lysates were fractionated by SDS-polyacrylamide gel electrophoresis and then the separated proteins were transferred onto polyvinylidene difluoride filters (Millipore, Bedford, MA, USA). The filters were incubated with primary antibodies against total immunoglobulin (Ig) (1:1000; Dako, Denmark), rabies virus nucleoprotein (1:5000; a gift from Dr. N. Minamoto, Gifu University) and alpha-tubulin (1:3000; Sigma, St Louis, MO, USA). After washing, the filters were incubated with horseradish peroxidase-conjugated secondary antibodies and signals were analyzed with an image analyzer (Fuji Film, Tokyo, Japan). For the isolation of Ig fractions, lysates were incubated with protein G sepharose beads (GE Healthcare Bio-Science Corp. Piscataway, NJ) and the isolated Ig fractions were subjected to neutralization assay, as described for the RFFIT.

2.5. Histopathology and immunohistochemistry

The brain, liver, spleen, kidneys, heart and lungs were collected and fixed in 20% neutral phosphate-buffered formalin. Paraffin embedded organs were cut into 4 μ m thick sections, and then the sections were stained with hematoxylin and eosin for light microscopy. For the detection of rabies viral antigen, the sections were stained using the streptavidin–biotin immunoperoxidase complex method (Histofine SAB-PO kit, Nichirei, Japan) and a rabbit anti-rabies virus polyclonal antibody (established in our laboratory). Sections were counterstained with Mayer's hematoxylin.

3. Results

3.1. Body weight changes, clinical signs and neutralizing antibody titers in blood

In SC immunized and control mice, mean body weight gradually decreased after virus inoculation, whereas there was no significant change in mean body weight in IC immunized mice (Fig. 1a). Additional symptoms included ruffled hair and loss of activity starting at 3 dpi, followed by progressive

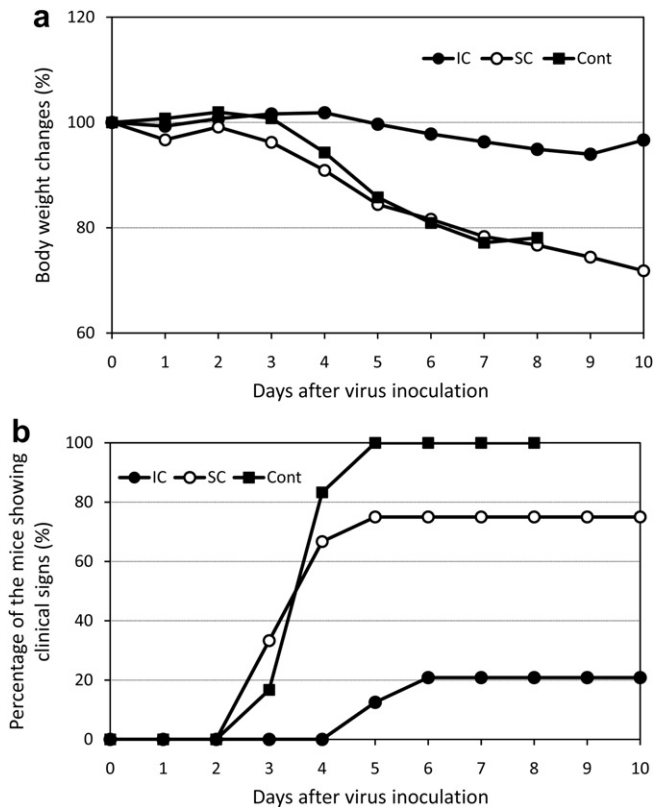


Fig. 1. Mean body weights (a) and percentages of animals exhibiting clinical signs (b) for intracerebrally (IC) immunization (closed circle), subcutaneous (SC) immunization (open circle) and control (closed box) group. Each group was composed of at least twelve mice. All control mice died before 8 dpi after exhibiting clinical signs; 75% and 20% of mice died in the SC and IC groups, respectively.

paralysis in the extremities and hypothermia in all of the mice in the control group, 75% of SC immunized animals and only 20% of IC immunized animals (Fig. 1b). All mice that exhibited clinical signs, regardless of group, died or reached a pre-determinant end-point for euthanasia. Virus neutralizing antibody titers of the IC group were significantly higher at pre-virus inoculation (Fig. 2a) and 3 dpi (Fig. 2b) as compared to the control and SC groups. The significant high antibody titer was still in 7 dpi in IC group compared to control (Fig. 3c). There was no significant difference between IC and SC group in 7 dpi.

3.2. Ig expression in each immunized mouse brain

The brains collected from systemically perfused mice were lysed and fractionated, and expression levels of total Ig were analyzed by Western blot. In the brains of IC immunized mice, heavy and light chains of Ig were detected (Fig. 3a, lane 3) but not in the brain of control and SC immunized mice (Fig. 3a, lanes 1 and 2). Further, Ig fraction isolated from the brain of IC immunized mice showed significant focus reduction *in vitro* compared to control and SC groups (Fig. 3b). Next, virus inoculated brain lysates from each treated mouse revealed that there was also a high level of induced Ig (Fig. 3c, lanes 2 and 5) in

IC immunized mice, and virus nucleoprotein was detected 3 and 7 dpi in control mice, and 7 dpi in SC immunized mice (Fig. 3c, lanes 1, 4 and 6).

3.3. Histopathology and immunohistochemistry in virus inoculated-mouse brain

Histopathological analysis of brain from control and SC immunized mice revealed evidence of mild non-suppurative encephalitis. We also observed pyknotic nuclei with vacuolation of neuropil, necrotic nerve cells with homogenous eosinophilic cytoplasm (Fig. 4a, b), and perivascular cuffing of mononuclear cells with marked activation of microglial cells (Fig. 4c) in control and SC immunized mice. None of these pathological changes were observed in the brains of IC immunized mice. Immunohistochemical analysis revealed RV antigens in the cytoplasm of nerve cells in control (Fig. 4d) and SC immunized mice (Fig. 4e). Positive signals were detected from 3 dpi in control and SC immunized mice and increased with time. Signals were present not only in cell bodies but also in nerve processes (Fig. 4e). In IC immunized mice, virus antigen was rarely detected in the brain, which was consistent with the results of Western blot analysis (see Fig. 3). However, a few positive signals were detected in nerve cell bodies in IC immunized mice at 5 dpi (Fig. 4f). The immunohistochemical results are summarized in Table 1. In addition, mild lymphoid cell depletion was observed in the spleens of mice that exhibited clinical symptoms, and there were no significant changes in any of the other organs.

4. Discussion

Many neurotropic viruses including herpes viruses, corona virus, Bornavirus, enterovirus and influenza virus, invade CNS from the periphery by transneuronal spread. RV is a typical neurotropic pathogen that is distributed worldwide [1]. Although it remains controversial whether or not RV replicates in the muscles at the site of exposure [17–19], the critical preventive measure is blocking neuroinvasion and transneuronal spread of the virus. We previously showed that IC immunization followed by challenge with an intramuscular inoculation of a lethal dose of RV survived without any neurological symptoms, and there was no evidence of viral replication in the spinal dorsal root ganglia neurons in these mice [14]. In the current study, we demonstrated that IC immunization induces neutralizing antibodies in the brain and prevents RV spread in the CNS.

The spread of RV within neurons occurs exclusively through retrograde fast axonal transport and trans-synaptic transport [19,20], and is mediated by virus glycoprotein [21]. Ultrastructural analysis indicates that most viral budding occurs on synaptic or adjacent plasma membranes [17]. There are at least two potential mechanisms of virus neutralization that would be consistent with the results of the current study. The first involves capture of inoculated virus by neutralizing antibodies induced in the CSF by IC immunization, before viral attachment and/or invasion of neurons, resulting in

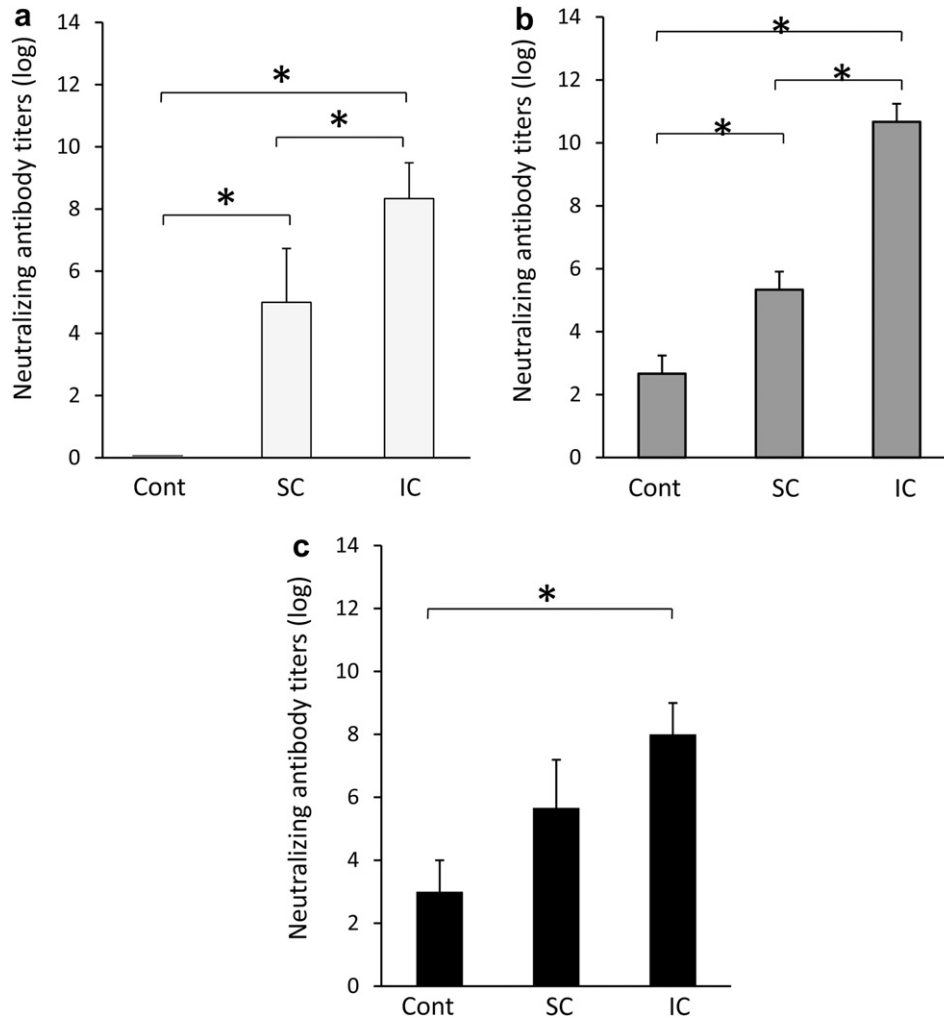


Fig. 2. Virus neutralizing antibody titers in blood plasma at pre-virus inoculation, 3 and 7 days post virus inoculation (dpi) in each immunized mouse. Antibody titers of the IC group were significantly higher at pre (a) and 3 dpi (b) compared to control and SC groups. The higher antibody titer was still significant in 7 dpi in IC group compared to control group (c). There was no significant difference between SC and IC group at 7 dpi. Bars indicate standard deviation and asterisks indicate statistical significance between groups ($p < 0.05$).

a dramatic decrease in infectious titer. However, immunohistochemical analysis indicated that at least some of the injected virus was able to infect neurons in IC immunized mice (Fig. 4). The other possibility is virus spread is inhibited by the neutralizing antibodies, which would mean that budding progeny virions are trapped by neutralizing antibodies before invading adjacent neurons. Although the precise mechanism has yet to be resolved, it is also possible that a combination of these two mechanisms contributes to the suppression of virus spread. Thus, the immune responses induced by IC immunization indicate that this approach may represent an effective therapeutic measure for CNS diseases caused by neurotropic pathogens.

Humoral immunity plays an essential protective role during the course of RV infection. Virus neutralizing antibodies that target virus glycoprotein play a critical role in protection mediated by T helper cells [22–25]. The absence of a conventional lymphatic system and the presence of a blood–brain barrier and blood–CSF barrier make the CNS an

immunologically privileged site. Recent reports however, have shown that immunization via the brain or CSF can elicit a systemic humoral immune response, characterized by production of antigen-specific antibodies in the cervical lymph nodes, spleen, serum and CSF [10,11,26]. Furthermore, intrathecal immunization via the subarachnoid or IC route yields a greater antibody response in the serum and CSF than extracerebral inoculation for a variety of inert antigens, including bacterial toxin and xenogenic albumin, and greater immunogenicity of CSF-administered antigen has been observed under a wide variety of conditions irrespective of the status of the blood–brain barrier (i.e. intact or disrupted blood–brain barrier) [10–14,26,27]. The CSF drains primarily into the blood, deep cervical lymph nodes and lymphatics located in the connective tissue sheaths of the peripheral nervous system, and partly into fluid spaces lying along the spinal nerve roots and peripheral nerves [27,28]. Thus, CSF is in direct contact with the extracellular fluid of the CNS, including synapses. The collection of CSF from mice in the current study was technically

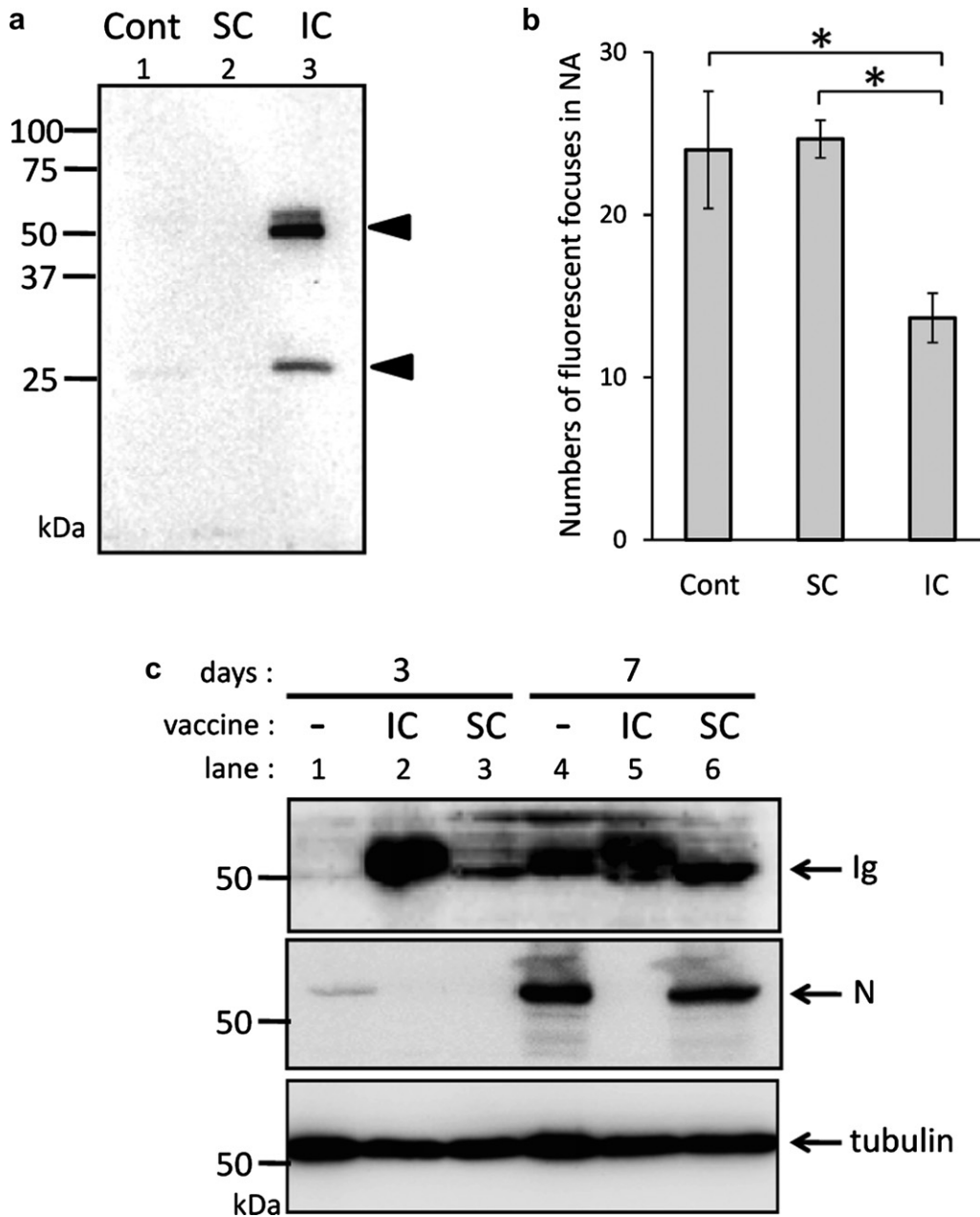


Fig. 3. Immunoglobulin (Ig) expression in the brains collected from systemically perfused mice (a). Heavy (upper arrow head) and light (lower arrow head) chains of Ig were detected only in the brain of IC immunized mice (a, lane3). These Ig fraction from IC immunized mice showed significant focus reduction in virus infected mouse neuroblastoma cell (NA) (b). Whole brain lysates from virus inoculated mice also showed high Ig induction in IC immunized mice (c, lanes 2 and 5). Middle panel also demonstrates proliferated viral nucleoprotein (N) in control mice at 3 and 7 days post virus inoculation (dpi) (lanes 1 and 4) and in SC immunized mice at 7 dpi (lane 6). Vaccine (–) indicates PBS inoculated mice (control group). Tubulin was equally detected in all lanes.

prohibitive; therefore, we estimated antibody production in the CNS by analyzing whole brain lysate (Fig. 3). Theoretically, these antibodies could be derived from two sources, plasma influx into the CNS across the blood–brain barrier, or antibody-secreting cells located within the CNS. We previously observed a marked increase in antibody titer in CSF in rabbits immunized with inactivated influenza virus via the subarachnoid [12], however we did not observed comparable changes in SC immunized rabbits, despite high antibody titers in blood serum. In additional experiments, more than 80% of mice died after intracerebral virus inoculation which were immunized with high

dose vaccine by SC route (200 µl, whereas 30 µl for IC immunization), and passive serum transfer also failed to rescue the rabid mouse (data not shown), suggesting the increased antibodies in blood could not work in the nervous tissues to suppress the viral spreading. Further, histopathological findings of perivascular edema or vasculitis were never observed in the brain of IC immunized mouse. We furthermore detected antibody-secreting cells in the brains of IC immunized mice by real-time RT-PCR and immunohistochemistry, but not SC immunized mice (unpublished data). These findings suggest a mechanism involving synthesis of antigen-specific antibodies within the

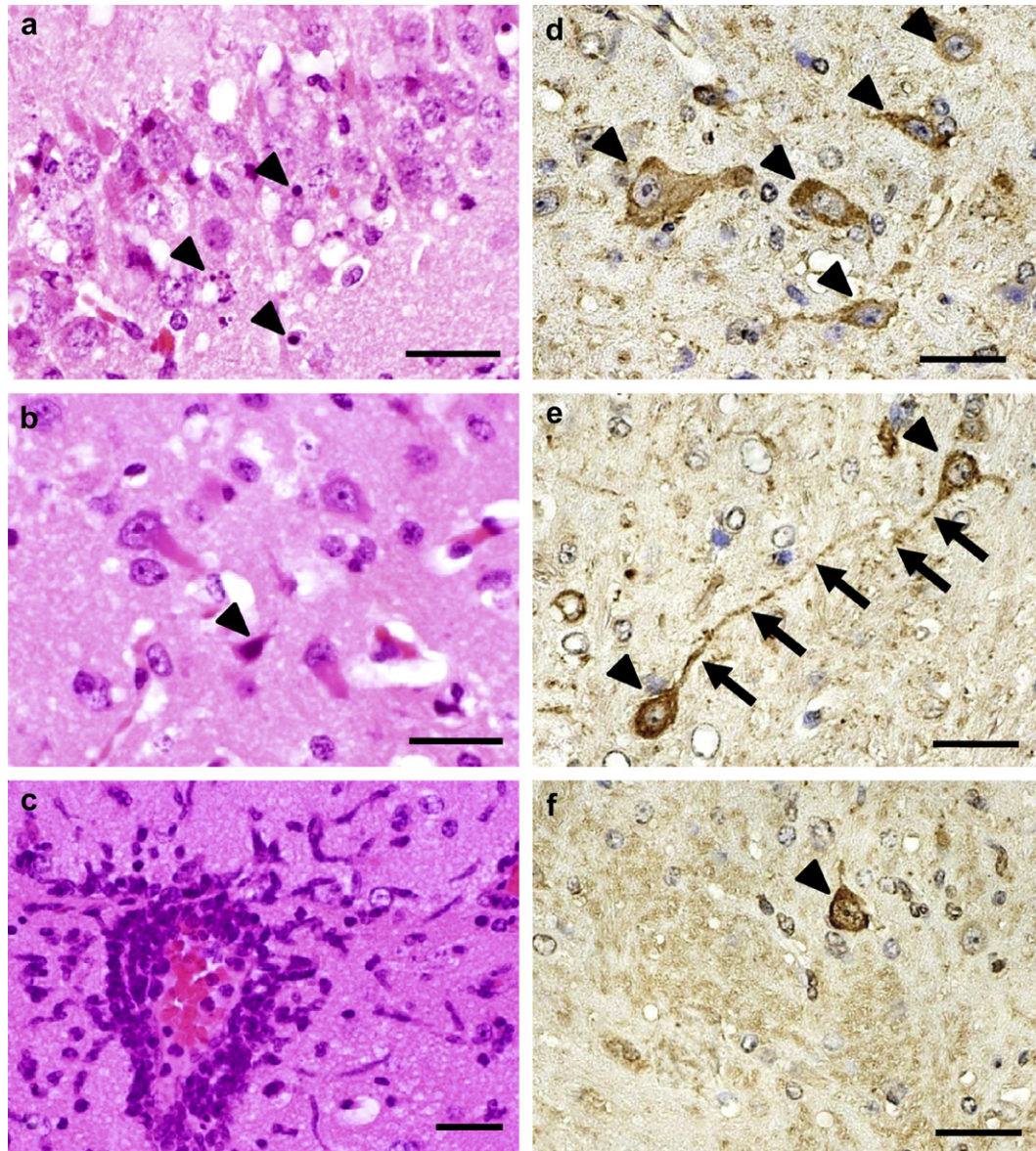


Fig. 4. Histopathology and immunohistochemistry of brain tissue following inoculation with rabies virus. There was evidence of mild non-suppurative encephalitis in rabid mouse brain (a–c). Pyknotic nuclei (arrow heads) and vacuolation (a, control, 6 dpi, hippocampus), necrotic nerve cells (arrow head in b, SC, 7 dpi, cerebral cortex), and perivascular cuffing of mononuclear cells with marked glial reactions (c, control, 7 dpi, hippocampus). Virus nucleoprotein was evident in perikaryon (arrow heads in d) and nerve processes (arrows in e) in control and SC mice, respectively (7 dpi, pons). Rabies virus antigen (arrow head) was also detected in some cells in the IC mouse brain (f, 5 dpi, thalamus). Bars are 30 μ m.

CNS. Recent reports also support the importance of antibody production by invading B cells in the clearance of RV from the CNS [29]. In conclusion, our results strongly indicate that IC

immunization induces a humoral immune response in mice that may play a role in suppressing the spread of RV in the brain.

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Table 1
Summary of the findings of IHC for rabies virus antigen in mice brain.

(dpi)	Control	SC	IC
3	++	+	–
5–7	+++	++	–/+
8–10	+++	+++	–

(+: low : ++: intermediate : +++: severe: – : not detected. dpi: days post virus inoculation.).

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